



## Engineering of Microbial Cell Factories for the Production of Plant Polyphenols with Health-Beneficial Properties

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1 **TITLE**

2 Engineering of Microbial Cell Factories for the Production of Plant Polyphenols with Health-Beneficial  
3 Properties

4 **RUNNING TITLE**

5 Microbial production of polyphenols

6

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23

24

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26 quercetin, resveratrol, *Saccharomyces cerevisiae*.

27 **Abstract**

28 Polyphenols form a group of important natural bioactive compounds with numerous ascribed health-beneficial  
29 attributes (*e.g.* antioxidant, anti-inflammatory, anti-microbial and tumor-suppressing properties). Some polyphenols  
30 can also be used as natural dyes or plastic precursors. Notwithstanding their relevance, production of most of these  
31 compounds still relies on extraction from plant material, which for most of it is a costly and an inefficient procedure.  
32 The use of microbial cell factories for this purpose is an emerging alternative that could allow a more efficient and  
33 sustainable production. The most recent advances in molecular biology and genetic engineering, combined with the  
34 ever-growing understanding of microbial physiology have led to multiple success stories. Production of multiple  
35 polyphenolic compounds or their direct precursors has been achieved not only in the common production hosts, such  
36 as *Escherichia coli* and *Saccharomyces cerevisiae*, but also in *Corynebacterium glutamicum* and *Lactococcus lactis*.  
37 However, boosting production of native compounds or introduction of heterologous biosynthetic pathways also  
38 brings certain challenges, such as the need to express, balance and maintain efficient precursor supply. This review  
39 will discuss the most recent advances in the field of metabolic engineering of microorganisms for polyphenol  
40 biosynthesis and its future perspectives, as well as outlines their potential health benefits and current production  
41 methods.

42

## 43 Introduction

44 There exist over 200,000 different secondary metabolites in plants [1, 2]. Of those, polyphenols are among the  
45 most widespread and ubiquitous classes. It has been estimated that in some cases up to 20% of the fixed carbon  
46 goes into the phenylpropanoid pathway that leads to the production of the majority of naturally-occurring phenolic  
47 compounds [3]. Although polyphenols are classified as secondary metabolites, i.e. molecules that in plants play little  
48 or no role in primary metabolism and therefore are not essential for cell's survival under normal conditions, these  
49 compounds may accumulate in considerably high amounts [4]. Polyphenols perform many diverse functions in  
50 plants, including anti-microbial and anti-fungal protection, insect feeding deterrence, providing coloration to leaves,  
51 flowers, and fruits, attraction of pollinators, chelation of toxic heavy metals, protection from UV radiation-induced  
52 damage, and free radical scavenging [5–8].

53 In plants, the aromatic amino acids *L*-phenylalanine and *L*-tyrosine are the two biosynthetic precursors of  
54 phenylpropanoid compounds (**Fig. (1)**), [9]). This group consists of compounds with the C<sub>6</sub>-C<sub>3</sub> backbone, such as  
55 cinnamic acid derivatives, coumarins, and lignans [10]. This backbone can be further extended with up to three two-  
56 carbon units derived from malonyl-CoA generating various polyphenols, such as curcuminoids, flavonoids,  
57 stilbenes, and styrylpyrones. Of those, the flavonoids (C<sub>6</sub>-C<sub>3</sub>-C<sub>6</sub> backbone) are the largest group. The vast chemical  
58 diversity of flavonoids arises from differences in the backbone structure, as well as from a variety of modifications  
59 to the backbone. Possible modifications are acetylation, aryl-migrations, glycosylations, hydroxylations,  
60 methylations, polymerizations, and prenylations. Based on these variations, the flavonoids can be further subdivided  
61 into aurones, flavanones, flavones, isoflavones, flavonols, flavan-3-ols, anthocyanidins, and tannins (**Fig. (1)**), [10]).  
62 A good example of the diversity of the decorations is provided by the anthocyanins, most of which are  
63 anthocyanidins glycosylated at the position 3 of the C-ring. Anthocyanins may have additional functional groups  
64 such as glycosyl groups (*e.g.* 5-glycosylation and 3'-glycosylation), methyl groups (*e.g.* 3'-methylated petunidin and  
65 7, 3'-methylated rosinidin), hydroxyl groups (which distinguish pelargonidin, cyanidin, and delphinidin derivatives),  
66 and acyl groups (on glycosyl moieties, could be both aromatic and/or aliphatic). These decorations can profoundly  
67 affect chemical properties such as color, hydrophobicity, and stability, as well as have a strong impact on the  
68 compounds bioavailability and bioactivity [11–14].

69 Polyphenols exhibit an immense natural chemical diversity and appear to have a number of different molecular  
70 targets, participating in several signaling pathways, and exhibiting pleiotropic activities both in plants and inside the  
71 human body when taken up as a part of diet [13, 15–20]. In addition, their occurrence in plants as complex mixtures  
72 makes it possible to take advantage of additive or synergistic activities of such combinations [21–23]. As a result of  
73 their diversity in structure and the ethnic knowledge of the use of particular plants in traditional medicine [24, 25],  
74 polyphenols have been the subject of intense research with respect to their health benefits [26–28]. Along with their  
75 use as pharmaceuticals, polyphenols have found many other potential applications such as natural pigments and food  
76 colorants, preservatives, monomers for bioplastics and composites, etc. [29–32].

77 In recent years the global market for polyphenols has seen continuous growth, with the main booster being the  
78 accumulated evidence of polyphenols' health-promoting traits leading to increased sales of polyphenols-containing  
79 food supplements, cosmetics, and other type of pharma- and nutraceutical products [33, 34]. In order to keep up with  
80 the increasing demand, there is a need for innovative solutions to the large-scale production of such compounds that  
81 can replace the less economic and eco-friendly traditional production methods, such as direct purification from plant  
82 raw materials or chemical synthesis. In this scenario, bio-based production of chemicals through metabolic  
83 engineering of microorganisms has emerged as a viable, affordable, and sustainable alternative. The advent of  
84 functional “Omics”, genome-scale modeling, and high-throughput screening technologies, combined with the ever-  
85 growing genome engineering, editing, and (heterologous) gene expression toolbox, has brought metabolic  
86 engineering to a more systematic and global level, thus significantly reducing the costs associated with the complete  
87 development of a novel bioprocess. The variety and complexity of chemicals that can now be produced using  
88 microbial cell factories has remarkably increased allowing now even the production of complex polyphenols with  
89 multiple biosynthetic pathways steps [35, 36].

90 This review will first focus on health benefits of different polyphenol groups and their applications. The current  
91 extraction and production approaches will then be discussed. Lastly, the ongoing research towards development of  
92 alternative production methods with a special emphasis on harnessing the potential of microbes as cell factories for  
93 the biosynthesis of polyphenols will be covered.

94

95 **Polyphenols, their health-beneficial effects, and potential applications**

96

97 Although primarily known as anti-oxidants, polyphenols possess multiple other health-beneficial properties. Most  
98 notably, there are several studies that recommend daily intake of high levels of polyphenols as a mean of reducing  
99 risks of cardiovascular disorders and type II diabetes [37–39]. Furthermore, these compounds were demonstrated to  
100 have an effect on certain types of cancer, neurodegenerative diseases, allergies, inflammation, and also to alleviate  
101 menopausal symptoms by estrogen mimicking [16, 40–44]. Additionally, polyphenols were documented to have  
102 anti-inflammatory, anti-aging, anti-angiogenic properties, as well as anti-viral and antimicrobial activities against  
103 human pathogens [45–50]. An overview of the specific health-promoting properties of the major groups of  
104 polyphenols is given in Table 1.

105

106 Bioactivities of polyphenols extend beyond the field of pharmaceuticals, and these compounds are also known for  
107 their many applications in food and cosmetics industries as natural substituents for synthetic ingredients. The  
108 concern over the safety of synthetic colorants raised by consumer groups [51, 52], combined with the health benefits  
109 provided by the many naturally-occurring pigments, has triggered the current marketing trend for replacement of  
110 artificial food dyes with natural colors [53]. In this respect, the bright and diverse colors of anthocyanins (hue range  
111 from red to bluish-red to purple), together with their health-beneficial properties, relative stability, and high  
112 solubility in water, make these flavonoids excellent candidates for food applications (Table 1) [12, 54–56]. As  
113 multiple anthocyanins are approved by the European Food Safety Authority [57], a great demand for their use as  
114 potential substitutes for the banned synthetic dyes is expected [58–60]. Similarly to colorants, the search for natural  
115 solutions to replace currently-used preservatives is also ongoing. Processed foods with minimal synthetic additives  
116 and thermal treatment are becoming an increasing trend. Thus, innovative solutions to extend product shelf-life,  
117 such as the use of natural preservatives, are required [61, 62]. The antimicrobial activity of flavonoids is a general  
118 attribute of this group of compounds, with some specific flavonoids also possessing anti-viral and anti-fungal  
119 properties [47, 63]. Moreover, being strong antioxidants [8, 64] flavonoids could potentially protect food from some  
120 undesirable chemical changes. Altogether, these attributes make flavonoids a very promising new group of natural  
121 preservatives suitable for use in food industry. Cosmetics is another area of interest for polyphenol applications.  
122 Multiple plant species that are enriched in polyphenols, such as cocoa, grape, olive, and tea, are used in cosmetic

123 products [65–67]. The potential of the application of coffee polyphenolic extract and of caffeic acid alone as  
124 components for skin care formulations has also been studied [68, 69]. Apart from protecting skin from UV radiation  
125 and reactive oxygen species, polyphenols have several other beneficial effects, including antiaging (via inhibition of  
126 lipooxygenase, cyclooxygenase, and skin re-modeling enzymes elastase and collagenase), depigmenting (via  
127 inhibition of tyrosinase), inhibition of inflammatory responses, and anti-microbial activity [66, 70, 71]. These data  
128 provide a good foundation for further research directed towards the application of purified polyphenolic compounds  
129 (single or as mixtures) in skincare products.

130

### 131 **Current approaches/methods for the production of polyphenols**

132

133 At the moment all purified polyphenolic compounds available on the market are either obtained through extraction  
134 from plant sources (*e.g.* fruits, leaves, or roots), or by total or partial chemical synthesis. So far, resveratrol remains  
135 the only exception to these traditional production methods that is also produced by microbial synthesis using  
136 *Saccharomyces cerevisiae* (Table 2). The extraction procedures impose numerous limitations that hinder the  
137 exploitation of polyphenols for pharmaceutical and biotechnological applications at their full potential [72, 73].  
138 Factors such as low natural abundance inside plant tissues, environmental and geographical conditions, seasonal  
139 variation, and the need for complex downstream processing could have a significant impact on the extraction yields  
140 of polyphenols. Consequently, the extraction procedures from plant sources are generally labor-intensive, costly, and  
141 using plant sources may consume large amounts of resources, such as water and land. Furthermore, the resulting  
142 preparations tend to contain impurities. One of the best known examples is the laxative emodin that is often co-  
143 purified with and contaminates resveratrol extracts from Japanese knotweed (*Polygonum cuspidatum* Siebold &  
144 Zucc.) [74]. Furthermore, despite all the progress made in the field of metabolic engineering of plants and plant cell  
145 factories for increased production of native secondary metabolites, their application as production hosts for  
146 polyphenols is still limited [75–79]. On the other hand, chemical synthesis of polyphenols has limited options for  
147 large-scale production due to the high structural complexity of these molecules. Both *de novo* synthesis and  
148 synthesis from purified precursors involves the use of hazardous and toxic chemical solvents, as well as extreme  
149 reaction conditions, thus limiting their application to specialized small-scale production [80]. Molecular chirality  
150 imposes additional challenges to chemical synthesis, as this process is not stereo-specific and yields a mixture of *R*-

151 and *S*-stereoisomers, whereas only 2*S*-stereoisomers of polyphenols were shown to be bioactive [81]. Consequently,  
152 an extra purification step is required for separating the isomers further reducing the final yield. Hence, more modern  
153 and environmentally-minded approaches are required in order to meet the growing demands for these  
154 phytochemicals.

155

### 156 **Microbial production of plant polyphenols: past achievements and ongoing research**

157

158 The inefficiency of traditional production methods is a major obstacle to broadening the range of applications for  
159 added-value polyphenols, and consequently successful commercialization would require implementation of large-  
160 scale, cost-effective, and sustainable production processes. In light of that, construction of designer microorganisms  
161 serving as biological platforms for the production of phenolic compounds is becoming a promising alternative.

162 Industrial workhorses, such as *Bacillus subtilis*, *Escherichia coli* or *Saccharomyces cerevisiae*, have been used for  
163 decades in numerous bioprocesses, including biological production of compounds with applications in

164 pharmaceutical, food, and chemical industries. Microbial production of fine chemicals presents multiple advantages  
165 that have been reviewed previously [82–84]. Briefly, as compared to plants and plant cell cultures, the

166 microorganisms that are used for production are usually fast-growing and easy to cultivate, which greatly reduces  
167 processes costs and production times. They are also able to grow in diverse media, including industrial and

168 agricultural waste, which makes the bioproduction more sustainable. Moreover, microbial fermentations are readily  
169 scalable from laboratory through demonstration to commercial production scales. Also, the ease of genetic

170 manipulation with these organisms and the availability of molecular tools (*e.g.* for expression of heterologous  
171 polyphenol pathway genes, for manipulation of homologous polyphenol pathway genes, or for genome editing)

172 facilitates the construction of microbial cell factories tailored for production of nearly any natural (and even non-  
173 natural) metabolite imaginable [83]. Furthermore, the use of microbial hosts for the production of polyphenols

174 simplifies the product purification procedure, as their secondary metabolism is generally much simpler and  
175 competing pathways can be eliminated or deactivated. Lastly, as opposed to the traditional methods for obtaining

176 natural products, microbial-based production can be a lot more environmentally-friendly, as the use of organic  
177 solvents or other harsh chemicals for product purification can be reduced [85, 86]. Production of fine chemicals



178 using microorganisms also requires considerably less natural resources, such as extensive land and water usage, as  
179 well as fertilizers and pesticides, needed to obtain and process large amounts of raw plant material [87, 88].

180  
181 Over the past years, multiple studies demonstrated the potential of microbial cell factories for the production of  
182 diverse classes of plant natural products (reviewed in [73, 83]). Among the polyphenols, various flavonoids [35, 83],  
183 stilbenes [89], raspberry ketone, a raspberry flavor molecule [90], caffeic acid, a lignin precursor [91, 92], and  
184 curcuminoids [93, 94] have been heterologously produced in microorganisms. The most interesting examples of  
185 polyphenol production using microbial cell factories are summarized in Table 3. There is also an example of  
186 production titers, productivity, and yield that meet the targets of the large-scale commercialization being achieved.  
187 The Swiss company Evolva is manufacturing resveratrol using yeast (*S. cerevisiae*) as the production platform [95].  
188 Up to now their EveResveratrol™ remains the only marketed phenolic compound produced *de novo* by fermentation  
189 in metabolically-engineered microorganisms (Table 2). It is also noteworthy that Evolva has either already  
190 established, or is about to initiate microbial production of several other phytochemicals, including the flavor and  
191 fragrance ingredient vanillin, the spice saffron, and the natural stevia sweeteners (<http://www.evolva.com/products/>).  
192 This example clearly demonstrates the feasibility of commercial bio-based production of plant-borne compounds in  
193 microbial cell factories.

194 From early days, the health benefits ascribed to polyphenols have prompted a significant amount of research work  
195 towards the elucidation of their biosynthetic pathways, the genes involved, and their regulation. The information  
196 gathered over the past years has led to multiple cases of successful genetic and/or metabolic engineering of whole  
197 plants or of plant cell cultures for improved biosynthesis of native and non-native polyphenolic compounds (for  
198 selected reviews see [33, 75, 77, 96–99]). This section, however, will only focus on the latest developments in the  
199 field of polyphenol production by microorganisms, since these are probably the better candidates for a large-scale  
200 and sustainable production.

201 The first steps of the phenylpropanoid biosynthesis pathway lead to the production of *p*-coumaric acid via  
202 deamination of the aromatic amino acids *L*-phenylalanine or *L*-tyrosine (**Fig. (1)**). Production of *p*-coumaric acid  
203 from *L*-phenylalanine is a two-step process where the amino acid is first converted into *trans*-cinnamic acid by a  
204 phenylalanine ammonia-lyase (PAL), which is further hydroxylated by cinnamate 4-hydroxylase (C4H), a

205 cytochrome P450 enzyme. The successful production of *p*-coumaric acid from *L*-phenylalanine was first  
206 demonstrated in *S. cerevisiae* by co-expression of PAL- and C4H-coding genes [100]. However, expression of this  
207 pathway in bacteria presents a challenge due to involvement of the P450 enzyme. These proteins are usually  
208 membrane-bound, thus functional expression in prokaryotes is difficult due to lack of the endoplasmic reticulum.  
209 Also, cytochromes P450 rely on P450 reductase enzymes (CPR) for cofactor regeneration, which are not present in  
210 bacteria and therefore need to be co-introduced into production strains [36]. In contrast, conversion of *L*-tyrosine into  
211 *p*-coumaric acid occurs in a single step catalyzed by a tyrosine ammonia-lyase (TAL), circumventing the need for  
212 C4H activity. A recent study describes several novel highly-specific TAL enzymes that are functional and produce  
213 high levels of *p*-coumaric acid in *E. coli*, *Lactococcus lactis*, as well as in *S. cerevisiae* [101]. Alternatively, use of a  
214 promiscuous PAL that can also take up *L*-tyrosine as a substrate for the production of the flavanone naringenin has  
215 been reported in *E. coli* [102, 103].

216 Flavonoids are by far the most explored group of polyphenols in terms of heterologous production in  
217 microorganisms [104]. *De novo* biosynthesis of complex flavonoids would require efficient production of the  
218 flavonoid core molecules, flavanones. These compounds are synthesized by CoA-esterification of cinnamates, such  
219 as *p*-coumaric acid and cinnamic acid, by 4-coumaroyl-CoA ligase (4CL), followed by condensation with three  
220 malonyl-CoA molecules catalyzed by chalcone synthase (CHS) and subsequent ring closure by chalcone isomerase  
221 (CHI). Further chemical modifications of flavanones, such as hydroxylations, methylations, methoxylations,  
222 acylations, and glycosylations, give rise to the vast diversity of flavonoid compounds (**Fig. (1)**). Flavanones such as  
223 naringenin and pinocembrin were successfully produced in *E. coli* and *S. cerevisiae* by co-expression of different  
224 combinations of PAL/TAL, 4CL, CHS, and CHI enzymes (for an overview see [35, 105–107]). Biosynthesis of a  
225 more complex flavanone, eriodictyol, has also been engineered in *E. coli* by additional expression of a flavonoid 3'-  
226 hydroxylase (F3'H) enzyme [108]. Furthermore, by combining 4CL, CHS, CHI, and chalcone reductase (CHR),  
227 liquiritigenin, 7-hydroxyflavanone, and butin were produced from, respectively, *p*-coumaric acid, cinnamic acid,  
228 and caffeic acid as precursors in both *E. coli* and *S. cerevisiae* [109]. To further broaden the spectrum of  
229 microbially-produced flavonoids, the biosynthesis of flavones [110, 111] and isoflavones [112] from precursors was  
230 achieved through the additional introduction of flavone synthase (FNS) or isoflavone synthase (IFS) genes,  
231 respectively. In another study, isoflavone genistein was produced directly from *L*-phenylalanine in yeast [107] and  
232 from *L*-tyrosine in *E. coli* [113]. Co-expression of the above-mentioned flavanone biosynthetic genes with flavanone

233 3-hydroxylase (F3H)- and flavonol synthase (FLS)-coding genes yielded the flavonols, kaempferol from  $L$ -tyrosine  
234 and galangin from  $L$ -phenylalanine [114]. Similarly, production of the flavonol fisetin from  $L$ -tyrosine has been  
235 recently established in *E. coli* by combining the liquiritigenin biosynthesis genes with the genes coding for F3H,  
236 FLS, and F3'H [115]. By combining F3H, dihydroflavonol reductase (DFR) and leucocyanidin reductase (LAR), the  
237 production of flavan-3-ols (+)-catechin and (+)-afzelechin was achieved from caffeic acid and *p*-coumaric acid,  
238 respectively [116]. Lastly, flavanones have also been converted to anthocyanins in a four-step pathway involving  
239 F3H, DFR, anthocyanidin synthase (ANS), and anthocyanidin 3-*O*-glucosyltransferase (3GT) [117, 118].

240 As mentioned above, flavonoids can be further modified by various decorating enzymes. Such modifications not  
241 only alter chemical properties and improve stability, but sometimes also grants the compounds novel biological  
242 activities [119, 120]. Thus, modified flavonoids might present additional commercial interest. The most common  
243 modification of plant flavonoids is glycosylation, often occurring at least at one position [120]. Multiple studies  
244 addressed the issues of glycosylation of flavonols [121, 122], flavones [123, 124], flavanones [125], and isoflavones  
245 [122, 126, 127]. Addition of other sugar moieties has been also successfully attempted, including rhamnosylation  
246 [121, 128, 129], xylosylation [130, 131], and galactosylation [132]. Lastly, biosynthesis of quercetin 3-*O-N*-  
247 acetylglucosamine has been reported as well [133].

248 Methylation is another common modification, and there have been several studies aiming at microbial biosynthesis  
249 of methylated flavonoids. One such example is the work of Kim *et al*, where *E. coli* strains for the production of  
250 ponciretin (4'-*O*-methylnaringenin) and sakuranetin (7-*O*-methylnaringenin) were constructed [134]. Other  
251 examples refer to the construction of strains producing the medically-important flavanonol 7-*O*-methyl  
252 aromadendrin from *p*-coumaric acid [135] and the flavonol genkwanin (7-*O*-methyl apigenin) from  $L$ -tyrosine.

253 Compared to flavonoids, microbial production of stilbenes is somewhat less of a hot topic. Nevertheless, numerous  
254 health benefits attributed to this group of polyphenols did stimulate research efforts to produce them heterologously  
255 in microorganisms for various applications in pharmaceutical and food industries. Similarly to flavonoids, stilbenes  
256 are produced via decarboxylative condensation of three malonyl-CoA molecules with the CoA-activated  
257 hydroxycinnamates through the action of stilbene synthase (STS) (**Fig. (1)**). Original attempts to establish microbial  
258 production of stilbene resveratrol were mainly done by the co-expression of the *4cl* and the *sts* genes, and have been  
259 accomplished in both *S. cerevisiae* and *E. coli* [136–140]. A more systematic approach comprising the use of two

260 different production strains, two promoter systems, screening of a *sts* gene library, and fine-tuning of gene  
261 expression levels further improved the production of resveratrol from *p*-coumaric in *E. coli* [141]. The engineered  
262 strain *E. coli* BW27784 expressing the *4cl* gene from *Arabidopsis thaliana* and the *sts* gene from *Vitis vinifera*  
263 organized in a bi-cistronic operon on a high-copy number plasmid, accumulated the impressive amount of 2.4 g/L  
264 resveratrol after the addition of a fatty acid biosynthesis inhibitor to improve precursor availability. Research efforts  
265 to bypass the use of the expensive precursor *p*-coumaric acid by supplying external L-phenylalanine or L-tyrosine  
266 resulted in consistently low titers of resveratrol [104, 107, 142, 143]. However, extensive strain optimization  
267 through i) increase of the availability of L-phenylalanine and malonyl-CoA, ii) integration of the resveratrol  
268 biosynthetic pathway in the genome and iii) introduction of a resveratrol exporter resulted in a *S. cerevisiae* strain  
269 capable of producing 4 g/L of resveratrol from glucose in a fed-batch fermentation [95]. More recently, resveratrol  
270 was also produced through *de novo* biosynthesis from both glucose and ethanol via the L-tyrosine intermediate at  
271 approximately 0.5g/L [144]. Biosynthesis of pinosylvin from L-phenylalanine has been also reported [145, 146].  
272 Several other studies focused on the production of methylated resveratrol derivatives, such as the mono-methylated  
273 pinostilbene and the di-methylated pterostilbene, which are equally or sometimes even considerably more bioactive  
274 than resveratrol [147]. Production of both pinostilbene and pterostilbene from *p*-coumaric acid was achieved by  
275 additional expression of stilbene *O*-methyltransferases (OMTs) genes from various sources in both *S. cerevisiae* and  
276 *E. coli* [148, 149]. Furthermore, another study has reported production of the unnatural stilbene methyl ethers by the  
277 expression of *Oryza sativa* OsOMT1 in *E. coli* [145].

278 The ever-increasing knowledge of specific pathways and the discovery of novel enzymes have contributed to the  
279 microbial production of difficult-to-synthesize polyphenols, such as certain phenolic acids or coumarins, whose  
280 biosynthesis involves production of cytochrome P450 enzymes or *O*-methyl-transferases. Identification of several  
281 bacteria-compatible hydroxylases that can replace *p*-coumarate 3-hydroxylase (C3H, a P450 enzyme) has made it  
282 possible to engineer artificial pathways for the biosynthesis of caffeic and ferulic acids from L-tyrosine or *p*-  
283 coumaric acid [91, 142, 150–152]. Maximal concentrations of 767 mg/L of caffeic acid and 196 mg/L ferulic acid  
284 were produced *de novo* by L-tyrosine overproducer strains of *E. coli* expressing, respectively, TAL and 4-  
285 hydroxyphenylacetate 3-hydroxylase [150] or TAL, C3H, and a caffeic acid methyltransferase (COM) [152].  
286 Production of plant-specific coumarins in bacteria has been also described [153]. At the first stage, *E. coli* strains  
287 were engineered to convert the phenylpropanoid acid precursors, *p*-coumaric acid and ferulic acid, into the simple

288 coumarins, umbelliferone (4.3 mg/L) and scopoletin (27.8 mg/L), respectively. Furthermore, these two coumarins  
289 were later-on produced *de novo* without the addition of any precursor after assembling the complete artificial  
290 biosynthetic pathway in *E. coli*. This pioneering study set the foundation for microbial production of more diverse  
291 coumarin molecules. Coumarins are components of various polymers [154] and were also demonstrated to have  
292 analgesic and anti-inflammatory properties [155], thus their production in microbial cell factories could also be of a  
293 commercial interest.

294 Although the use of *S. cerevisiae* and *E. coli* presents multiple advantages, other microorganisms might be more  
295 suitable for production of polyphenolic compounds, for example due to higher end-product tolerance or a broader  
296 range of growth substrates. One of the first attempts of using a non-conventional host for the flavonoid production  
297 was done in *Streptomyces venezuelae*, where the flavanones naringenin and pinocembrin, as well as the stilbenes  
298 resveratrol and pinosylvin were produced from, *p*-coumaric acid and cinnamic acid, respectively [156]. The same  
299 organism was later on engineered for the biosynthesis of kaempferol and galangin by the co-expression of the *f3h*  
300 gene from *Citrus siensis* and the *fls* gene from *Citrus unshiu* and feeding with naringenin or pinocembrin,  
301 respectively [157]. A more recent study has demonstrated the feasibility of polyphenol production in  
302 *Corynebacterium glutamicum*, a soil bacterium that is used for amino acid production on industrial scale.  
303 Kallscheuer *et al*, were able to engineer this bacterium to produce resveratrol and naringenin directly from glucose  
304 with yields comparable to those observed in *E. coli* [158]. Moreover, *C. glutamicum* was further engineered for the  
305 production of resveratrol from 4-hydroxybenzoic acid (HBA) by reversal of a  $\beta$ -oxidative phenylpropanoid  
306 degradation pathway [159]. This allows having polyphenol production independent from the aromatic amino acid  
307 biosynthesis. The most “exotic” case was a study where the edible macrofungus *Tremella fuciformis* Berk. (silver  
308 ear or white jelly mushroom, division Basidiomycota) was genetically modified for the biosynthesis of resveratrol  
309 from *p*-coumaric acid with yields of 0.8-0.9  $\mu\text{g/g}$  of dry weight after 7 days of cultivation [160].

### 310 **Strategies for improving production of polyphenolic compounds in microorganisms**

311 Metabolic engineering is the introduction of targeted adjustments to cellular metabolic processes aiming at  
312 improving production of a certain substance. This is often achieved with a set of genetic manipulations that leads to  
313 alterations within various regulatory, enzymatic, and transport functions of the cell [161, 162]. Metabolic  
314 engineering is particularly important for optimization of heterologous pathways, as introduction of such pathways

315 often leads to flux imbalance, not only within the pathway, but also often within the global cellular metabolism. This  
316 occurs because the host generally lacks the regulatory machinery required for efficient and balanced operation of the  
317 pathway, and also to prevent over- or under-production of enzymes, leading to metabolic burden on the host at the  
318 cost of productivity of the compound of interest, as well as accumulation of potentially toxic intermediates [163].

319 One of the key targets for metabolic pathway engineering is the improvement of precursor supply. The most notable  
320 strategies for that are summarized in Table 4. Numerous studies dealing with polyphenol production have concluded  
321 that increasing the intracellular pool of malonyl-CoA is the key requirement for enhancing the flavonoid and  
322 stilbene production [112, 164, 165]. The most common strategies for that are a) the overexpression of an acetyl-CoA  
323 carboxylase (ACC)-coding gene that converts acetyl-CoA into malonyl-CoA and b) the inhibition of fatty acid  
324 biosynthesis by addition of the antibiotic cerulenin [35, 146, 166, 167]. Further improvement could be achieved  
325 through the fine-tuning of the acetate assimilation via the overexpression of acetyl-CoA synthetase gene and the  
326 deletion of the acetate-utilizing pathways, which overall resulted in a 16.3-fold increase of intracellular malonyl-  
327 CoA concentration [108, 166]. Other alternatives include the introduction of a malonate catabolic pathway [168,  
328 169], the overexpression of the 3-ketoacyl-ACP synthase genes *fabH* and *fabF* [166, 170], and the conditional  
329 down-regulation of fatty acid biosynthesis with CRISPR interference (CRISPRi) [171]. Several other studies in *E.*  
330 *coli* have taken a more global approach, combining computational predictions and experimental validations [165,  
331 172, 173]. The utilized genome-scale models predicted a set of genetic interventions, mainly aiming to up-regulate  
332 some of the glycolytic reactions and down-regulate the tricarboxylic acid (TCA) cycle that cooperatively drive the  
333 carbon flux towards malonyl-CoA, while at the same time preventing the formation of byproducts. These  
334 interventions were experimentally validated using a lab-scale fermenter, and the introduced genetic modifications  
335 resulted in a significantly improved production of naringenin (474 mg/L, [172]) and resveratrol (1600 mg/L, [173]).

336 Other substrates/co-factors critical for the biosynthesis of flavonoids are the UDP-glucose and NADPH [117, 169,  
337 174]. The former one is the donor of the glucosyl group in the anthocyanin biosynthesis as well as in other flavonoid  
338 biosynthetic routes, whereas the latter one is required for the biosynthesis of leucoanthocyanidins, 5-  
339 deoxyflavanones, and (+)-catechins (**Fig. (1)**). Engineering of UDP-glucose levels through a combination of  
340 overexpressing genes of the UTP biosynthetic pathway, supplementation with orotic acid, and deletion of several  
341 endogenous UDP-glucose-utilizing genes resulted in the significantly improved anthocyanin production from

342 flavan-3-ols precursors in an *E. coli* strain expressing ANS- and 3GT-coding genes [169]. Recently, a follow-up  
343 study has reported that overexpression of the *ycjU* gene, which catalyzes the conversion of  $\beta$  anomer of glucose-6-  
344 phosphate into glucose-1-phosphate, further increases the UDP-glucose pools, and consequently anthocyanin  
345 production [118]. With regard to improving the intracellular NADPH availability, a stoichiometry-based model was  
346 deployed to identify a set of potential gene knock-out combinations in *E. coli*. Upon validation of the candidates, the  
347 combined inactivation of phosphoglucose isomerase, phosphoenolpyruvate carboxylase, and phospholipase  
348 activities resulted in a 4-fold increase of leucoanthocyanidin production and a 2-fold increase of (+)-catechin  
349 production, as compared to the wild-type background [174].

350 Another challenge to the current metabolic engineering strategies is the use of media supplemented with expensive  
351 precursors (*e.g.* *p*-coumaric acid or naringenin) and, a two step-fermentation process (biomass/protein production  
352 and polyphenol synthesis) that becomes a disadvantage when the process scale-up is considered. Partly, this issue  
353 comes from the fact that biosynthetic pathways for complex polyphenols such as flavonols and anthocyanins consist  
354 of six or more genes. Overexpression of such high number of genes would first of all cause a large metabolic burden  
355 to the cell. This problem has already been partly addressed by using a co-culture strategy [175]. With this approach,  
356 Jones *et al.* have split the (+)-afzelechin biosynthetic pathway between the two co-incubated strains: the first one  
357 was expressing the malonyl-CoA-dependent part (from *p*-coumaric acid to naringenin), whereas the second strain  
358 was expressing the NADPH-dependent downstream part (from naringenin to (+)-afzelechin). Another advantage of  
359 the co-cultivation system is that the two strains could be independently engineered for enhanced precursor supply  
360 (*e.g.* malonyl-CoA or NADPH) without significantly impacting the cellular metabolism. One of the major  
361 parameters that need to be considered and optimized in such experiments is strain compatibility. The selected strains  
362 must have similar growth kinetics to avoid out competition of one, which can lead to an imbalance in the pathway.  
363 The authors addressed this issue by introducing the plasmids containing the biosynthetic genes into multiple  
364 background strains and selecting the most fitting combination. Equal growth could also potentially be ensured by  
365 introducing two different auxotrophies in the production strains in a way that would make them dependent on one  
366 another. Furthermore, production of compounds that are toxic to other members of the consortium must be avoided  
367 and efficient transfer of pathway metabolites from one partner to another must be ensured. The second issue is that  
368 expression of this many genes could be challenging due to lack of compatible sets of overexpression vectors for  
369 many industrially-relevant microorganisms. This, however, is not an issue for *E. coli* where the complete

370 biosynthetic pathway for the flavonol fisetin from *L*-tyrosine consisting of seven genes has been established using  
371 the DUET vector system, in which genes were expressed in pairs utilizing four different expression vectors [115].  
372 There is also an interesting solution in *S. cerevisiae* that involves the use of a polyprotein system allowing co-  
373 transcription of multiple coding sequences from a single promoter. The system has already been used for the  
374 production of 2-hydroxynaringenin-*C*-glucoside [176]. Furthermore, in order to allow complete *de novo*  
375 biosynthesis, both bacterial and yeast strains have been engineered for the production of flavonoids and stilbenes  
376 from inexpensive substrates, such as glucose, by introducing heterologous genes coding for various polyphenol  
377 biosynthesis pathways into *L*-tyrosine- or *L*-phenylalanine-overproducing strains [168, 177–179].

378 Microbial production of polyphenols is often challenged by toxicity of the end-product and/or of its biosynthetic  
379 intermediates, as well as by the formation of inclusion bodies resulting from protein overproduction. The former  
380 issue is related to anti-microbial properties of polyphenols, which could become an issue particularly if the produced  
381 molecules accumulate intracellularly at high concentrations. One possible approach to resolve this is to co-express  
382 an exporter protein that would extrude the produced polyphenols into the culture medium [95]. Another approach is  
383 to use adaptive laboratory evolution (ALE), which consists of continuous cultivation of the producer microorganism  
384 while subjecting it to increasing concentrations of the polyphenols. This process generally results in accumulation of  
385 mutations that would increase tolerance of the producer strain towards the target compound [180, 181]. Furthermore,  
386 cytotoxic effect of the biosynthetic intermediates could be avoided by balancing expression levels of individual  
387 genes within the given pathway [168, 182]. The second issue arises from the necessity for some enzymes to form  
388 complexes in order to ensure high local substrate concentrations, in particularly if a reaction is unfavorable [82].  
389 However, this could also be of an advantage even if the coupling is unnatural, as it would ensure efficient flux from  
390 one step of a pathway to another. There are multiple ways of ensure close proximity of biosynthetic enzymes and  
391 their intermediates, including intracellular compartmentalization [82], use of synthetic scaffolds [183], and  
392 construction of translational fusions. The latter approach has been used multiple times for engineering of polyphenol  
393 production, including construction of the 4CL::STS fusion for enhancing resveratrol production [137], construction  
394 of CHS::CHR fusion for increasing liquiritigenin production [115], and use of P450::CPR to allow functional  
395 expression of P450 enzymes in bacteria [108, 112, 115].



396 A recent report has drawn the attention to the interference of native aromatic acid degradation pathways with the  
397 production of polyphenols [135]. Detailed analysis of *E. coli* strains producing 7-*O*-methyl aromadendrin showed  
398 that the final concentration of this flavanonol did not correspond to the consumption of *p*-coumaric acid, indicating  
399 possible degradation of the precursor via an unknown pathway [184]. *S. cerevisiae* has been also reported to have  
400 more than one enzyme catalyzing decarboxylation of *trans*-cinnamic acid and *p*-coumaric acid, a reaction which  
401 could also potentially reduce production of polyphenols [185]. A similar situation was observed in *C. glutamicum*  
402 where a phenylpropanoid degradation gene cluster had to be deleted prior to engineering of this bacterium for  
403 stilbene and flavonoid production [158, 186]. Therefore, possible presence of such enzymes and pathways needs to  
404 be accounted for, in particular prior to exploration of a new production host.

405 Another interesting recent development is the emergence of combinatorial gene expression techniques that appear to  
406 be promising approaches to address the challenge of improving titers and productivity efficiency [163, 187].  
407 However, their successful application is highly dependent on the availability of high-throughput methods for strain  
408 screening [188]. Recently, two flavonoid biosensors were constructed consisting of the reporter gene coding for the  
409 fluorescent protein placed under control of the flavonoid-responsive transcriptional regulator [189]. The  
410 transcriptional regulators FdeR from *Herbaspirillum seropedicae* SmR1 was used to generate a biosensor to detect  
411 naringenin, whereas QdoR from *B. subtilis* was used to detect quercetin and kaempferol. The QdoR-based biosensor  
412 was highly efficient in detecting kaempferol production *in vivo* at the single cell level while using fluorescence-  
413 activated cell sorting (FACS). The developed biosensors could be subsequently used for identification of novel  
414 genes involved in polyphenol biosynthetic pathways [189]. Another biosensor has been developed based on the *B.*  
415 *subtilis* transcription factor FapR that is responsive to malonyl-CoA [190]. This sensor could therefore be used for  
416 selection of candidates with increased intracellular concentrations of malonyl-CoA. Liu *et al.* [191] have used the  
417 same transcription factor in order to develop a negative feedback regulatory circuit. The circuit relies on a malonyl-  
418 CoA-based sensor-actuator system that controls expression of the *acc* gene, and in this way alleviates the toxic  
419 effects of high intracellular concentration of the enzyme. The circuit was proven to be efficient in regulating the  
420 fatty acid biosynthetic pathway and increasing fatty acid titer and productivity [191]. Application of such system for  
421 microbial production of polyphenols should allow balancing the engineered pathways and subsequently improve the  
422 production efficiency. Other approaches and techniques that have been successfully utilized for fine-tuning gene  
423 expression for the needs of metabolic engineering were thoroughly reviewed in [192]. Furthermore, a new screening

424 technique based on high-performance thin-layer chromatography (HPTLC) has been developed for the discovery of  
425 flavonoid-modifying enzymes [193]. The authors claim that this metagenome extract thin-layer chromatography  
426 analysis (META) allows rapid detection of glycosyltransferases and other flavonoid-decorating enzymes, as well as  
427 that the system is highly sensitive, being able to detect of as little as 4 ng of a modified molecule.

## 428 **Conclusion**

429 There has been a substantial progress in the field of microbial production of polyphenols. The recent advances in  
430 genome editing, combined with novel engineering tools, now allow the expression of multiple genes coding for  
431 enzymes forming complex biosynthetic polyphenol pathways not only in model organisms such *E. coli* and *S.*  
432 *cerevisiae*, but also in more novel productions hosts, such as *C. glutamicum*, *L. lactis*, and *Streptomyces venezuelae*.  
433 Nonetheless, in many cases the production efficiency using microbial hosts remains inferior as compared to  
434 extraction from plants. However, constant advances of synthetic biology tools combined with future metabolic  
435 engineering efforts will further facilitate the development of more economically-favorable production processes.

## 436 **Abbreviations**

437 3GT – anthocyanidin 3-*O*-glycosyltransferase, 4CL – 4-coumaroyl-CoA ligase, AAT – anthocyanin acyltransferase,  
438 ACC – acetyl-CoA carboxylase, ALE – adaptive laboratory evolution, AMT – anthocyanin methyltransferase, ANR  
439 – anthocyanidin reductase, ANS – anthocyanidin synthase (leucoanthocyanidin dioxygenase), C3H – *p*-coumarate 3-  
440 hydroxylase, C4H – cinnamate 4-hydroxylase, CDW – cell dry weight, CHI – chalcone isomerase, CHR – chalcone  
441 reductase, CHS – chalcone synthase, CoA – Coenzyme A, COM – caffeic acid methyltransferase, CPR –  
442 cytochrome P450 reductase, CRISPRi – clustered regularly-interspaced short palindromic repeats interference, CUS  
443 – curcuminoid synthase, CVD – cardiovascular diseases, DFR – dihydroflavonol 4-reductase, F3'H – flavonoid 3'-  
444 hydroxylase, F3H – flavanone 3-hydroxylase, FACS – fluorescence-activated cell sorting, FLS – flavonol synthase,  
445 FNS – flavone synthase, HBA – 4-hydroxybenzoic acid, HTC – high-throughput screening, IFS – isoflavone  
446 synthase, K/O – knock-out, LAR – leucoanthocyanidin reductase, NADPH – nicotinamide adenine dinucleotide  
447 phosphate, O/E – overexpression, OMT – 3-*O*-methyltransferase, LDL – low-density lipoprotein, PAL –  
448 phenylalanine ammonia-lyase, STS – stilbene synthase, TAL – tyrosine ammonia-lyase, TCA cycle – tricarboxylic  
449 acid cycle, UDP-glucose – uridine diphosphate glucose.

450

451 **Conflict of Interest**

452 The authors declare no conflicts of interest.

453

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- 1023

1024 **Table 1. Health-beneficial properties of the major groups of polyphenols.**

<b>Polyphenol</b>	<b>Applications: health-promoting or biotechnological</b>
<p><b>Phenolic acids</b></p> <p><i>Examples:</i></p> <p>Gallic acid</p> <p>Caffeic acid</p> <p>Ferulic acid</p> <p>Chlorogenic acid</p>	<p><b>Cancer:</b> Chemopreventive activity, as well as protection against side effects of chemotherapy [194, 195].</p> <p><b>CVD and Diabetes:</b> Prevent oxidation of low-density lipoprotein (LDL)-cholesterol and effective in the treatment of hypercholesterolemia and type 2 diabetes [196–198].</p> <p><b>Neurodegenerative diseases:</b> Potential as agents for the treatment of Alzheimer’s disease [199].</p> <p><b>Others:</b> Anti-allergic; anti-microbial; antioxidant and immunomodulatory activities. Di- and tri-caffeic/quinic acids have antiretroviral activity [200–204].</p>
<p><b>Anthocyanins</b></p> <p><i>Examples:</i></p> <p>Cyanidin</p> <p>Pelargonidin</p> <p>Delphinidin</p> <p>Rosinidin</p>	<p><b>Cancer:</b> Inhibit initiation and progression stages of tumor development; reduce effect of inflammation on promotion of tumorigenesis; suppress angiogenesis; minimize cancer-induced DNA damage [205–207].</p> <p><b>CVD and Diabetes:</b> Improve vascular health; protect against cardiovascular diseases; anti-obesity effects through improvement of adipocyte function; may contribute to prevention of the metabolic syndrome; potential anti-diabetic activity [64, 205, 208–210].</p> <p><b>Neurodegenerative diseases:</b> Protection against brain ageing and decline in cognitive performance in animal models [205, 211, 212].</p> <p><b>Others:</b> Reduce inflammatory biomarkers; bacteriostatic against some gut pathogenic bacteria; food colorants [205, 208, 213, 214].</p>
<p><b>Flavonols</b></p> <p><i>Examples:</i></p> <p>Quercetin</p> <p>Kaempferol</p> <p>Myricetin</p> <p>Fisetin</p> <p>Morin</p>	<p><b>Cancer:</b> Protective effects against pancreatic, breast, cervical, prostate, uterine, urinary tract cancers, and leukemia [49, 215–217].</p> <p><b>CVD and Diabetes:</b> Confer cardioprotection and improve the levels of risk factors for cardiovascular disease [218, 219].</p> <p><b>Neurodegenerative diseases:</b> Neuroprotective activity in experimental focal ischemia and models of neurodegeneration; cognition-enhancing; reduce the risk of Alzheimer's disease [220–222].</p>

Rutin	<p><b>Inflammation:</b> Anti-inflammatory [223].</p> <p><b>Others:</b> Anticonvulsant, antioxidants, memory enhancement [203, 224, 225].</p>
<p><b>Flavanols</b></p> <p><i>Examples:</i></p> <p>Catechins</p> <p>Epigallocatechin</p> <p>Thearubigins</p> <p>Mesquitol</p>	<p><b>Cancer:</b> Inhibition of tumorigenesis in different organs of animals [205, 226].</p> <p><b>CVD and Diabetes:</b> Cardioprotective effect by reverting of endothelial dysfunctions; decreasing inflammatory biomarkers, and providing antioxidant and antiplatelet effects. Also have beneficial effects on blood pressure, blood glucose level, and lipid parameters [227–230].</p> <p><b>Neurodegenerative diseases:</b> Neuroprotective/neuroregenerative effects as modulators of intracellular neuronal signaling and metabolism, cell survival/death genes, and mitochondrial function [212, 231].</p>
<p><b>Hydrolyzable tannins</b></p> <p><i>Examples:</i></p> <p>Grandinin</p> <p>Casuarictin</p> <p>Punicalagin</p> <p>Vescalagin</p>	<p><b>Cancer:</b> Anti-tumor, anti-proliferative and anti-mutagenic effects [232, 233].</p> <p><b>CVD and Diabetes:</b> Anti-diabetic; anti-atherogenic; anti-thrombotic [234, 235].</p> <p><b>Others:</b> Anti-inflammatory, anti-bacterial, and anti-mycotic properties. Ellagitannins and gallotannins may also affect the life of foodstuff due to their antioxidant properties and/or antimicrobial activity [236, 237].</p>
<p><b>Isoflavones</b></p> <p><i>Examples:</i></p> <p>Genistein</p> <p>Daidzein</p> <p>Curcumin</p> <p>Glycetin</p>	<p><b>Cancer:</b> Inhibition of cell proliferation [47, 238, 239].</p> <p><b>CVD and Diabetes:</b> Anti-platelet effects [240, 241].</p> <p><b>Others:</b> Neuroprotective agents; improve cognitive functions and alleviate menopause symptom in females; anti-thyroid activity [242–244].</p>
<p><b>Proanthocyanidins</b></p> <p><b>(Condensed tannins)</b></p> <p><i>Examples:</i></p> <p>Epicatechin trimer</p> <p>Selligueain A</p> <p>Procyanidin B3</p>	<p><b>Cancer:</b> Reduce the incidence and progression of cancer (particularly of prostate cancer) [245, 246].</p> <p><b>CVD and Diabetes:</b> Reduction of CVD incidence due to their antioxidant activity; inhibition of LDL oxidation; vasodilating properties; anti-platelet activity and protection against ischemia-reperfusion injury [245, 247, 248].</p> <p><b>Others:</b> Proanthocyanidin-rich extracts inhibit viral adhesion and infectivity of the A</p>

	and B influenza viruses, as well as suppress urinary and <i>Helicobacter pylori</i> infections, procyanidin B3 has been described as a hair-growth stimulant, selligueain A is a natural sweetener [245, 249–251].
<b>Lignans</b> <i>Examples:</i> Secoisolariciresinol Pinoresinol Podophyllotoxin Steganacin	<b>Cancer:</b> Anti-carcinogenic effects on multiple types of cancer [252–254]. <b>CVD and Diabetes:</b> Associated with a decreased risk of cardiovascular diseases, hypoglycemic properties [255, 256]. <b>Others:</b> Inhibition of <i>H. pylori</i> motility and steroid hormone metabolism, anti-viral activities [252, 254, 257].
<b>Stilbenoids</b> <i>Examples:</i> Resveratrol Pterostilbene Pinosylvin Piceid	<b>Cancer:</b> <i>in vitro</i> as well as <i>in vivo</i> chemopreventive and chemotherapeutic activities, in all three stages of carcinogenesis (initiation, promotion, and progression) [46, 258]. <b>CVD and Diabetes:</b> Improve insulin sensitivity, mimics calorie restriction, lower plasma lipoproteins and cholesterol, prevent cell damage induced by oxidative stress and ischemia [259–262]. <b>Others:</b> anti-aging and anti-inflammatory activities, relieves endotoxemia-associated adrenocortical insufficiency, confer protection against intestinal barrier dysfunction, modulate gut microbiota by favoring increase in lactic acid bacteria counts [46, 214, 263–266].

1025 CVD – cardiovascular diseases

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1027 **Table 2. Examples of polyphenol-containing products accessible to consumers.**

Type of polyphenol	Production method	Examples of products on the market (supplier)
<b>Phenolic acids</b> (Hydroxycinnamic and hydroxybenzoic acids)	<ul style="list-style-type: none"> <li>• Extraction from plants</li> </ul>	<ul style="list-style-type: none"> <li>• GCA™-Green Coffee Antioxidant (Applied Food Sciences)</li> </ul>
<b>Anthocyanins</b>	<ul style="list-style-type: none"> <li>• Extraction from plants</li> <li>• Extraction from bilberry</li> <li>• Extraction from bilberry</li> </ul>	<ul style="list-style-type: none"> <li>• Freeze Dried Polyphenol Fruitbasket (BerryPharma)</li> <li>• Mirtoselect® and Myrtocyan® (Indena®)</li> <li>• NutriPhy® Bilberry 100 (Chr. Hansen A/S)</li> </ul>
<b>Flavonols</b> (Quercetin/kaempferol/myricetin)	<ul style="list-style-type: none"> <li>• Chemical synthesis</li> <li>• Extraction from plants</li> </ul>	<ul style="list-style-type: none"> <li>• Quercetin complex (Solgar)</li> <li>• Bayberry Bark Extract Myricetin (Cactus Botanics)</li> </ul>
<b>Flavanols</b> (Catechins)	<ul style="list-style-type: none"> <li>• Extraction from plants</li> <li>• Extraction from plants</li> <li>• Extraction from plants</li> </ul>	<ul style="list-style-type: none"> <li>• Green Tea Catechins, Decaf - <i>Camellia sinensis</i>, (Amax)</li> <li>• NutraSource, AssuriTEA Green, (Kemin Health)</li> <li>• Theaflavin Black Tea Extract (Applied Food Sciences)</li> </ul>
<b>Hydrolyzable tannins</b> (Casuarictin)	<ul style="list-style-type: none"> <li>• Extraction from plants</li> </ul>	<ul style="list-style-type: none"> <li>• PomActiv™ Pomegranate Extract (Cyvex Nutrition)</li> </ul>
<b>Isoflavones</b> (Genistein)	<ul style="list-style-type: none"> <li>• Extraction from soy</li> </ul>	<ul style="list-style-type: none"> <li>• geniVida® (DSM)</li> </ul>
<b>Proanthocyanidins</b> (Epichatechin trimer)	<ul style="list-style-type: none"> <li>• Extraction from plants</li> </ul>	<ul style="list-style-type: none"> <li>• Pine Bark 95% Proanthocyanidins (Cactus Botanics)</li> </ul>



	<ul style="list-style-type: none"> <li>• Extraction from plants</li> </ul>	<ul style="list-style-type: none"> <li>• ENOVITA® - grape seed extract and proanthocyanidin A2 phytosome (Indena)</li> </ul>
<b>Lignans</b> (Secoisolariciresinol)	<ul style="list-style-type: none"> <li>• Extraction from plants</li> <li>• Extraction from plants</li> </ul>	<ul style="list-style-type: none"> <li>• Flaxseed Lignans (Cactus Botanics)</li> <li>• ActiFlax (Marco Hi-Tech)</li> </ul>
<b>Stilbenes</b> (Resveratrol)	<ul style="list-style-type: none"> <li>• Extraction from plants</li> <li>• Chemical synthesis</li> <li>• Microbial production</li> </ul>	<ul style="list-style-type: none"> <li>• Rexatrol® - resveratrol phytosome® (Indena)</li> <li>• ResVida (DSM)</li> <li>• EveResveratrol™ (Evolva)</li> </ul>

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1030 **Table 3. Production of polyphenolic compounds in microbial hosts.**

Compound	Precursor	Host	Reference	Highest titer
<b>Phenolic acids</b>				
<i>p</i> -coumaric acid	L-Phenylalanine	<i>S. cerevisiae</i>	[100]	~ 7.2 mg/L
	L-Tyrosine	<i>S. cerevisiae</i>	[179]	1.93 g/L
	L-Tyrosine	<i>E. coli</i>	[101, 152]	1.6 mmol/g CDW
		<i>S. cerevisiae</i>	[101]	133 µmol/g CDW
		<i>L. lactis</i>		43 µmol/g CDW
Caffeic acid	L-Tyrosine	<i>E. coli</i>	[151, 152]	150 mg/L
	Glucose	<i>E. coli</i>	[91, 150]	767 mg/L
Ferulic acid	L-Tyrosine	<i>E. coli</i>	[152]	196 mg/L
<b>Flavanones</b>				
Naringenin	L-Tyrosine	<i>E. coli</i>	[102, 103, 164]	57 mg/L
	L-Phenylalanine	<i>S. cerevisiae</i>	[107]	8.9 mg/L
	<i>p</i> -Coumaric acid	<i>E. coli</i>	[172]	474 mg/L
	Glucose	<i>E. coli</i>	[178]	84 mg/L
	Glucose	<i>C. glutamicum</i>	[158]	32 mg/L
	Glucose	<i>S. cerevisiae</i>	[177]	113 mg/L
	<i>p</i> -Coumaric acid	<i>St. venezuelae</i>	[156]	4 mg/L
Pinocembrin	L-Phenylalanine	<i>E. coli</i>	[102, 164]	58 mg/L
	Glucose	<i>E. coli</i>	[168]	40 mg/L
	Cinnamic acid	<i>St. venezuelae</i>	[156]	6 mg/L
Eriodictyol	L-Tyrosine	<i>E. coli</i>	[108]	43 mg/L
Liquiritigenin	<i>p</i> -Coumaric acid	<i>E. coli</i>	[109]	17 mg/L
		<i>S. cerevisiae</i>		14 mg/L

7-hydroxyflavanone	Cinnamic acid	<i>E. coli</i>		1.9 mg/L
		<i>S. cerevisiae</i>		0.9 mg/L
Butin	Caffeic acid	<i>E. coli</i>		4.2 mg/L
		<i>S. cerevisiae</i>		2.5 mg/L
Sakuranetin	L-Tyrosine	<i>E. coli</i>	[134]	40 mg/L
Ponciretin	L-Tyrosine	<i>E. coli</i>	[134]	43 mg/L
<b>Flavones</b>				
Chrysin	Cinnamic acid	<i>S. cerevisiae</i>	[110]	0.9 mg/L
	L-Phenylalanine	<i>E. coli</i>	[114]	9 mg/L
Apigenin	<i>p</i> -Coumaric acid	<i>S. cerevisiae</i>	[110]	0.4 mg/L
	L-Tyrosine	<i>E. coli</i>	[111, 114]	30 mg/L
Genkwanin	L-Tyrosine	<i>E. coli</i>	[111]	41 mg/L
Luteolin	Caffeic acid	<i>S. cerevisiae</i>	[110]	1.6 mg/L
<b>Isoflavones</b>				
Genistein	Naringenin	<i>E. coli</i>	[112]	10 mg/g CDW
	L-Phenylalanine	<i>S. cerevisiae</i>	[107]	0.1 mg/L
Daidzein	Liquiritigenin	<i>E. coli</i>	[112]	18 mg/g CDW
<b>Flavonols</b>				
Kaempferol	L-Tyrosine	<i>E. coli</i>	[114]	15 mg/L
	Naringenin	<i>St. venezuelae</i>	[157]	0.2 mg/L
	L-Phenylalanine	<i>S. cerevisiae</i>	[107]	1.3 mg/L
Galangin	L-Phenylalanine	<i>E. coli</i>	[114]	1.1 mg/L
	Pinocembrin	<i>St. venezuelae</i>	[157]	1.0 mg/L
Fisetin	L-Tyrosine	<i>E. coli</i>	[115]	0.3 mg/L

Quercetin	<i>p</i> -Coumaric acid	<i>S. cerevisiae</i>	[107]	0.26 mg/L
7-O-methyl aromadendrin	<i>p</i> -Coumaric acid	<i>E. coli</i>	[135]	3 mg/L
<b>Flavan-3-ol</b>				
(+)-catechin	Caffeic acid	<i>E. coli</i>	[116]	0.09 mg/L
(+)-afzelechin	<i>p</i> -Coumaric acid	<i>E. coli</i>	[116]	0.04 mg/L
<b>Anthocyanins</b>				
Pelargonidin 3- <i>O</i> -glucoside	Naringenin	<i>E. coli</i>	[267]	6 µg/L
Cyanidin 3- <i>O</i> -glucoside	Eriodictyol	<i>E. coli</i>	[267]	6 µg/L
	(+)-catechin	<i>E. coli</i>	[118]	350 mg/L
<b>Stilbenes</b>				
Resveratrol	<i>p</i> -Coumaric acid	<i>S. cerevisiae</i>	[136–138, 140]	391 mg/L
	Glucose	<i>S. cerevisiae</i>	[144]	531 mg/L
	Glucose	<i>S. cerevisiae</i>	[95]	4 g/L
	<i>p</i> -Coumaric acid	<i>E. coli</i>	[139, 141, 173]	1600 mg/L
	Glucose	<i>C. glutamicum</i>	[158]	59 mg/L
	<i>p</i> -Coumaric acid	<i>St. venezuelae</i>	[156]	0.4 mg/L
	<i>p</i> -Coumaric acid	<i>T. fuciformis</i>	[160]	0.8 µg/g CDW
Pinosylvin	L-Phenylalanine	<i>E. coli</i>	[145, 146]	91 mg/L
	Glycerol	<i>E. coli</i>	[171]	47 mg/L
	L-Phenylalanine	<i>St. venezuelae</i>	[156]	0.6 mg/L
Pterostilbene	<i>p</i> -Coumaric acid	<i>E. coli</i>	[148]	50 mg/L
Pinostilbene	<i>p</i> -Coumaric acid	<i>E. coli</i>	[149]	34 mg/L

1031 CDW – cell dry weight

1032 **Table 4: Metabolic engineering strategies used for improving precursor supply for polyphenol biosynthesis**

Target	Approach	Host organism	References
<b>Malonyl-CoA pool</b>			
	Addition of cerulenin	<i>E. coli</i>	[146, 170, 173, 178]
		<i>C. glutamicum</i>	[158]
	O/E of ACC	<i>E. coli</i>	[108, 166, 172]
		<i>S. cerevisiae</i>	[144]
	O/E of acetyl-CoA synthase	<i>E. coli</i>	[108, 166]
	K/O of acetate kinase	<i>E. coli</i>	[108, 166]
	O/E of <i>fabF</i> and <i>fabE</i>	<i>E. coli</i>	[166, 170]
	Expression of MatB and MatC from <i>Rhizobium trifolii</i>	<i>E. coli</i>	[168, 169]
	Repression of <i>fabD</i>	<i>E. coli</i>	[171]
	Up-regulation of glycolysis and down-regulation of the TCA cycle	<i>E. coli</i>	[165, 172, 173]
<b>UDP-glucose availability</b>			
	O/E of <i>pgm</i> , <i>galU</i> , <i>ndk</i>	<i>E. coli</i>	[169]
	K/O of <i>galE</i> and <i>galT</i>	<i>E. coli</i>	[169]
	O/E of <i>ycjU</i>	<i>E. coli</i>	[118]
<b>NADPH availability</b>			
	Deletion of <i>pgi</i> , <i>ppc</i> , and <i>pldA</i>	<i>E. coli</i>	[174]
<b>Aromatic amino acid availability</b>			

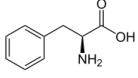
	Modifications of the shikimate pathway	<i>E. coli</i>	[168]
		<i>S. cerevisiae</i>	[177, 179]
	Reducing flux through the Ehrlich pathway	<i>S. cerevisiae</i>	[177]

1033 O/E – overexpression, K/O – knock-out, TCA cycle – tricarboxylic acid cycle

1034

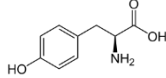
# Amino acid metabolism

L-Phenylalanine



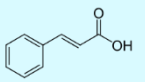
PAL

L-Tyrosine



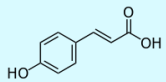
TAL

Cinnamic acid



C4H

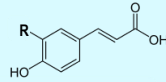
*p*-Coumaric acid



C3H

OMT

Other phenylpropanoids

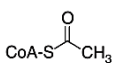


e.g. Caffeic acid

PHENOLIC ACIDS & Derivatives

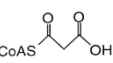
Sugar metabolism

Acetyl-CoA



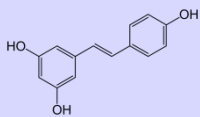
ACC

Malonyl-CoA



STS

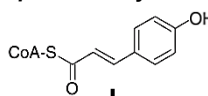
STILBENES



e.g. Resveratrol

4CL

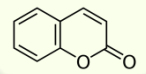
*p*-Coumaroyl-CoA



LIGNIN  
LIGNANS

PHENYLPROPENES

COUMARINS



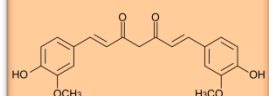
CHS

Chalcone

AURONES

CUS

CURCUMINOIDS

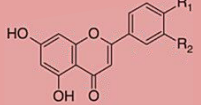


e.g. Curcumin

CHI

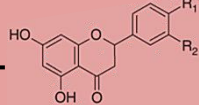
FLAVONOIDS

Flavones



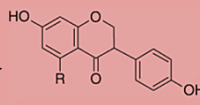
e.g. Apigenin

Flavanone



e.g. Naringenin

Isoflavones



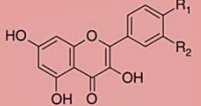
e.g. Genistein

FNS

IFS

Isoflavonoids

Flavonols



e.g. Quercetin

F3H

FLS

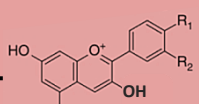
Dihydroflavonol

DFR

Leucoanthocyanidins  
(Flavan-3,4-diols)

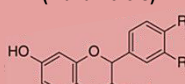
ANS

Anthocyanidins



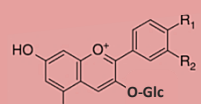
e.g. Pelargonidin

Catechins  
(Flavan-3-ols)



e.g. (+)-catechin

Anthocyanins



e.g. Chrysanthemine

3GT

AAT

AMT

ANR

Proanthocyanidins  
(Condensed tannins)

**Figure 1.** Plant polyphenols and their biosynthetic routes. Names of enzymes: 3GT, anthocyanidin 3-*O*-glycosyltransferase; 4CL, 4-coumaroyl-CoA ligase; AAT, anthocyanin acyltransferase; AMT, anthocyanin methyltransferase; ANR, anthocyanidin reductase; ANS, anthocyanidin synthase (leucoanthocyanidin dioxygenase); C3H, *p*-coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; CHI, chalcone isomerase; CHS, chalcone synthase; CUS, curcuminoid synthase; DFR, dihydroflavonol 4-reductase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; FNS, flavone synthase; IFS, isoflavone synthase; LAR, leucoanthocyanidin reductase; OMT, 3-*O*-methyltransferase; PAL, phenylalanine ammonia-lyase; STS, stilbene synthase; TAL, tyrosine ammonia-lyase ([1–3]).

- [1] Falcone Ferreyra ML, Rius SP, Casati P. Flavonoids: biosynthesis, biological functions, and biotechnological applications. *Front Plant Sci* 2012; 3: 222.
- [2] Katsuyama Y, Kita T, Funa N, et al. Curcuminoid biosynthesis by two type III polyketide synthases in the herb *Curcuma longa*. *J Biol Chem* 2009; 284: 11160–70.
- [3] Vogt T. Phenylpropanoid Biosynthesis. *Mol Plant* 2010; 3: 2–20.